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13. SUPPLEMENTARY NOTES

14. ABSTRACT Heretofore, efforts to develop implantable sensors for real-time clinical monitoring of glucose subcutaneously (SQ) in diabetic patients have been hindered by the unreliable analytical results owing largely to biocompatibility problems induced by sensor implantation (e.g., inflammatory/foreign body response). The goal of this research program is to explore and optimize the chemistries required to fabricate implantable amperometric glucose sensors with outer polymeric coatings that slowly generate low levels of nitric oxide (NO). Release of NO has been shown to enhance the biocompatibility of the implanted sensors by decreasing the inflammatory response. The focus of this research has been to develop new polymeric coatings (biomedical hydrogels and polyurethanes) that possess immobilized copper ion sites or organoselenium and organotellurium species that will serve as catalytic sites for *in situ* conversion of endogenous nitrosothiol species (RSNO) to NO, thereby providing a sustained local generation of NO species at the surface of the implanted sensors. Preliminary biocompatibility experiments suggest that RSNO levels within the SQ fluid of rats may be sufficient to generate enough local NO to reduce the inflammatory response at the implant site. New needle type sensors are being developed to determine the levels of RSNOs in the SQ region. Finally, functional needle type SQ glucose sensors have been prepared with both NO release and NO generation coatings. These sensors provide the basis of assessing if NO generation/releasing chemistries are compatible with glucose sensing chemistries.

15. SUBJECT TERMS
Biocompatibility; sensors; inflammation; angiogenesis; polymeric coatings; nitric oxide

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Table of Contents

INTRODUCTION

To date, efforts to develop implantable sensors for real-time clinical monitoring of glucose subcutaneously (SQ) in diabetic patients have been limited by the unreliable analytical results that occur largely due to biocompatibility problems induced by sensor implantation (e.g., inflammatory/foreign body response). The goal of this research program is to explore and optimize the chemistries required to fabricate implantable amperometric glucose sensors with outer polymeric films that slowly generate low levels of nitric oxide (NO) from endogenous S-nitrosothiol (RSNO) species that are likely present in the interstitial fluid. The local generation of NO is expected to greatly enhance the biocompatibility of the implanted sensors by decreasing the inflammatory response and promoting angiogenesis as well as wound healing at the implant site.

Although our previous studies [1] with thin NO release polymer coatings on SQ glucose sensors have already demonstrated that local NO release can reduce initial inflammatory response of the surrounding SQ tissue, the reservoir of NO precursors that can be retained in such thin polymeric coatings is low and this makes it difficult to achieve prolonged NO release (>1 d) at physiologically relevant levels. Hence, for long-term sensor implants (weeks to months), a completely new strategy to generate NO locally at the surface of the devices may well be required. The primary focus of this research program is to develop new polymeric coatings (biomedical hydrogels and polyurethanes) that possess immobilized copper (II) ion or other sites (e.g., organoselenium or organotellurium) that will serve as catalytic surfaces for *in situ* conversion of any endogenous nitrosothiol species (e.g., nitrosoglutathione, nitrosocysteine, etc.) to NO, thereby providing a sustained local generation of NO species at the surface of the implanted sensors. Experiments are being undertaken to assess whether these new NO generating polymers placed within the SQ fluid of rats can decrease inflammatory response. Finally, functional needle type SQ glucose sensors are being prepared that possess appropriate analytical performance for glucose measurements. These functional sensors will be used as the basis to ensure that the NO generating chemistry is compatible with the sensing chemistry and that its function will not be compromised by the use of these novel biocompatible coatings. Simultaneously, we are also revisiting the NO release polymer strategy, with a new sensing configuration that has the potential to enable longer-term NO release, without compromising glucose response characteristics.

BODY

Steady progress has been made over the past year toward most of the goals in the approved Statement of Work for this project, with the ultimate aim of solving the biocompatibility problem for SQ implanted electrochemical glucose sensors. Our three main goals are: 1) to prepare and characterize new polymers (derivatized polyurethanes and poly(hydroxyethylmethacrylates)) possessing immobilized copper(II) ion sites (via Cu(II)-cyclen complexes) and potentially other catalysts such as organoselenium and organtellurium species that can generate NO from RSNO species as a next generation of anti-inflammatory coatings; 2) *in vivo* testing of NO generation and concomitant anti-inflammatory response within subcutaneous tissue of rats with sham sensor devices coated with polymers containing the novel NO generating catalysts, and 3) fabricate functional needle-type glucose sensors in our laboratory with the new NO generation (and also new longer-term NO release) coatings, and demonstrate that NO generation/release chemistry does not decrease the analytical functionality of such glucose sensing devices. We are also interested to prepare needle type sensors that will be able to detect the levels of RSNO species in the SQ fluid, to ensure that levels are adequate for the NO generating concept to become a potential solution to the overall biocompatibility problem. Results during the past year of research are summarized below.

1) Synthesis and Characterization of New Polymers/Coatings that Catalytically Generate NO: As reported last year, we developed a new NO generating poly(HEMA) material with immobilized Cu(II)-cyclen sites that is capable of generating NO from various RSNO species [2]. This past year, we successfully prepared a new polyurethane material with covalently linked Cu(II)-cyclen species, and demonstrated that this new polymer can

also generate NO from various RSNOs. The synthetic route used for this new PU material is outlined in Figure 1.

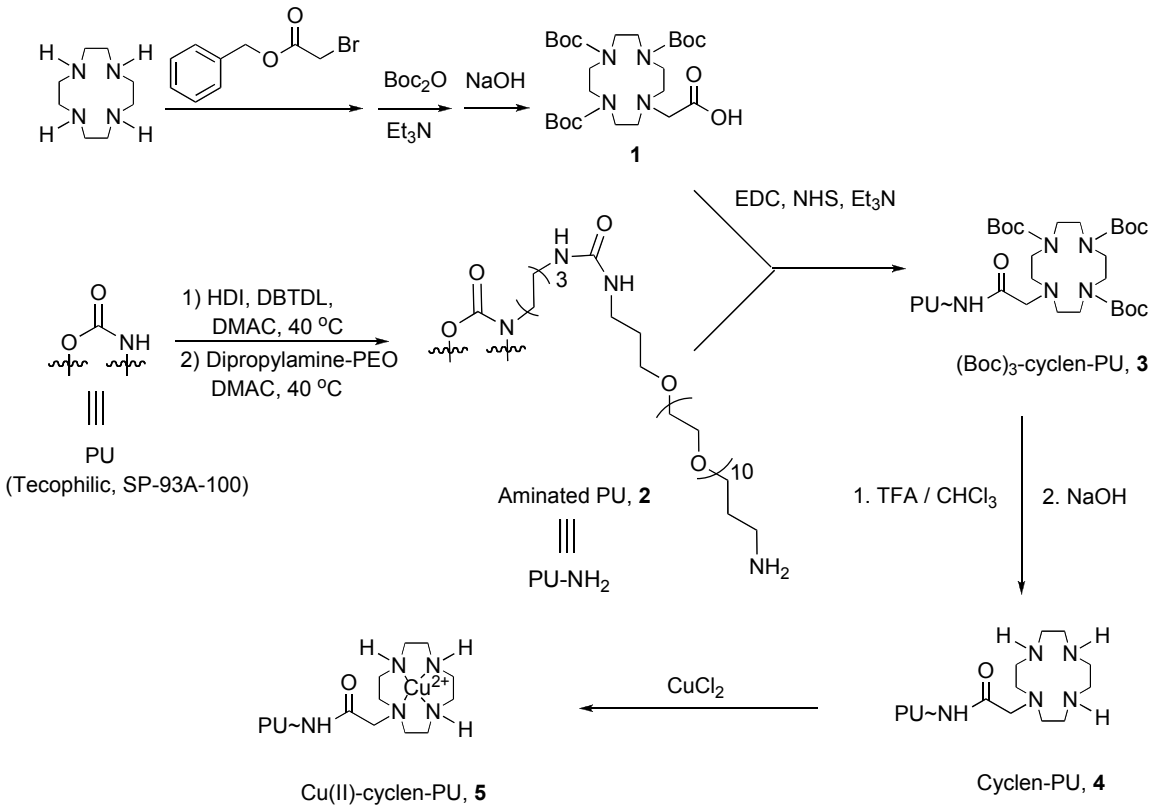

Figure 1. The synthesis of a polyurethane possessing covalently appended Cu(II)-cyclen complex (Cu(II)-cyclen-PU (**5**)) from a modified cyclen derivative (**1**) and aminated PU (**2**).

We employed an existing medical grade hydrophilic thermoplastic PU (Tecophilic®, SP-93A-100) as the primary polymer which was then aminated (**2**) for attachment of $(Boc)_3$-cyclen-N-acetic acid (**1**). After the conjugation via EDC coupling chemistry, the Boc-groups on the cyclen were deprotected with TFA and the mixture was treated with dilute NaOH to remove TFA. Copper ions were then incorporated into the polymer. Extensive washing procedures with various solvents were used to remove any non-specifically bound Cu(II) ions and ultimately afforded the desired polymer, Cu(II)-cyclen-PU (**5**). Resulting polymer **5** had copper content, as determined by ICP-Mass Spectrometry, in the range from 0.08 to 0.4 wt %, depending on the specific reaction conditions employed.

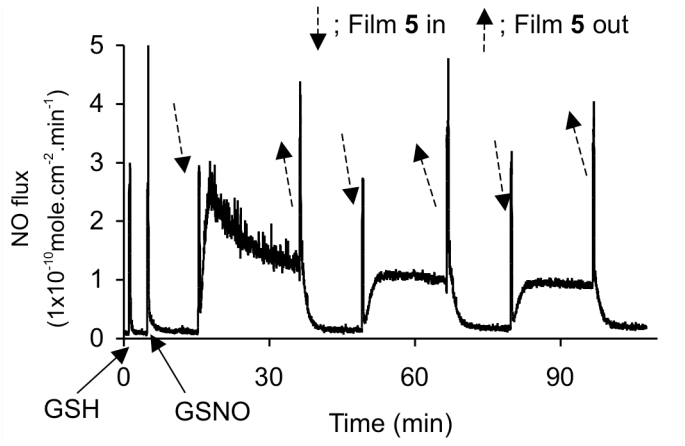

Figure 2. NO generation from piece of Cu(II)-cyclen-PU material when placed into solution of nitrosoglutathione/glutathione (GSNO/GSH) as measured by chemiluminescence detection of NO.

As shown in Figure 2, when a small disk of a polymer **5** is placed into a solution of 10 μM GSNO/GSH in phosphate buffered saline (PBS), pH 7.4, a burst of NO is detected (see Figure 2) and then the NO flux slowly decreases to reach a steady-state NO level (approximately 1 x 10^{-10} mole/cm^2.min). When the film is removed from the solution, the NO signal returns to the original baseline, implying that the presence of this polymer initiates the NO liberation from GSNO (nitrosoglutathione). The repeated insertion/removal of

the film demonstrates that the polymer can generate a comparable steady-state NO flux after each immersion and removal from the test solution (see Figure 2). Therefore, this new polymer represents a promising material for coating implantable glucose sensors.

Despite success in preparing polymers with immobilized Cu(II)-ligand sites, we have found a few potential shortcomings in using such Cu(II) catalysts. One major concern is that these catalysts have very low rates of reaction with S-nitrosoglutahione (GSNO) compared to S-nitrosocysteine (CysNO). Hence, if GSNO levels are equal to or greater than CysNO in the SQ fluid (concentrations unknown at this point), less total NO may be generated on polymer interfaces that have Cu(II) sites vs. some other catalyst that possesses equal reactivity with all RSNO species. Toward this end, we recently began work on with immobilized organoselenium (RSe) and organotellurium (RTe) species as potential surfaces to generate NO from endogenous RSNOs [3,4]. Both types of catalysts have been found to generate NO from all RSNOs as similar rates. Further, these same catalytic surfaces can be utilized to create novel electrochemical RSNO sensors [5-7] for measurement of RSNOs in blood and potentially in SQ fluid (one of goals for this project).

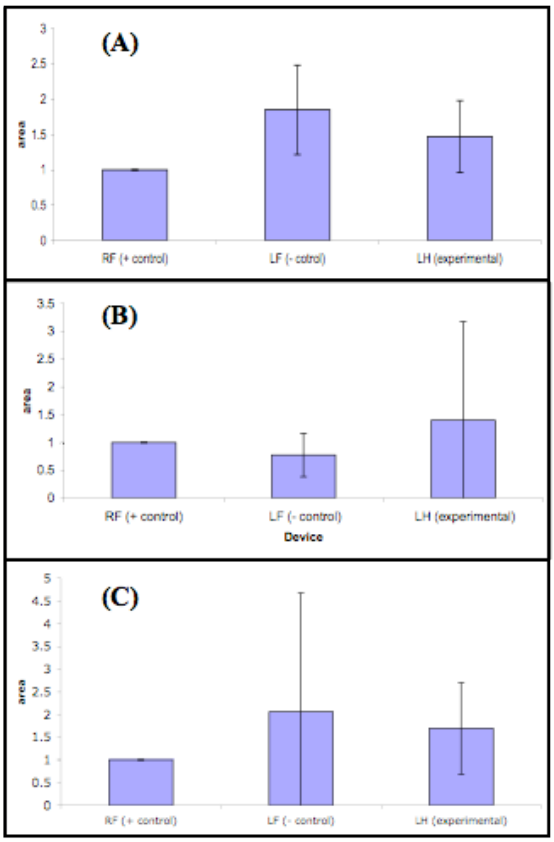

Figure 3. Results from implant pathology where devices released NO (+ control) or generated NO (experimental) compared to plain polymer coatings (- control). The area plotted on the y-axis is the ratio of the surface area of inflammatory cells/total cells observed. Results were standardized to the + control within each animal. (A) Figure 1 reproduced from last year's progress report (n = 8). (B) The result from this year's experiment (n = 10). (C) Recalculated results of this year's experiment assuming the implants were mislabeled.

One novel approach to utilize these new RSe and RTe catalysts for coatings on implantable glucose sensors involves the development of a layer-by-layer (LbL) deposition method, to make it convenient to coat the catalysts on any sensor surface material. The LbL method requires that the catalyst be attached to a highly charged polyion polymer. In our initial studies, we have utilized polyethylenimine (PEI) as the polymer and covalently linked RSe sites to this polymer. This species is then alternately dip coated with a polyanionic polymer, alginate, to create the LbL coating that is held in place by strong electrostatic interactions between the RSe-PEI and the alginate. The coatings have been found to be reasonably stable, and can generate more NO from RSNOs if a greater number of layers are coated on a given substrate. A manuscript describing this new technique is now in the preparation stage. The LbL method will be attractive for coating glucose sensors with an NO generating surface.

2) *In Vivo* Testing of NO Generation from Subcutaneous Tissue in Rats: We reported in the last progress report that initial *in vivo* testing of sham devices (n = 10) coated with a Cu(II)-cyclen complex blended into PurSil (a biomedical grad polyurethane) showed reduced inflammatory response similar to that expressed by the positive control (NO release). Figure 1 from last year's report is reproduced here as Figure 3A. The data is standardized to the inflammatory response of the positive NO release control within each animal. Therefore, a lower value translates to improved biocompatibility. Unfortunately, when we tried to repeat this experiment again, the data was not in agreement with that of the first set. This is shown in Figure 3B. However, we suspect that this could be due to at least some of the implants being mislabeled. This is because when the data is recompiled assuming that the lowest inflammation always comes from positive controls (NO release coatings and data renormalized to this average) and the experimental (NO generation) and negative control data are

switched (due to mislabeling), the data closely resembles that from the first set of data (Figure 3A). This is shown in Figure 3C. If some of the implants were mislabeled, this would also explain the larger deviation observed in this year's data. While we cannot be certain what is the true reason for the disagreement in the data, we are in the process of repeating this experiment for a third time (n = 4) and are awaiting the pathology report for this follow-up experiment. Nonetheless, we have reproduced, now in more than 19 animals, that NO release coatings clearly reduce inflammatory response in every experiment.

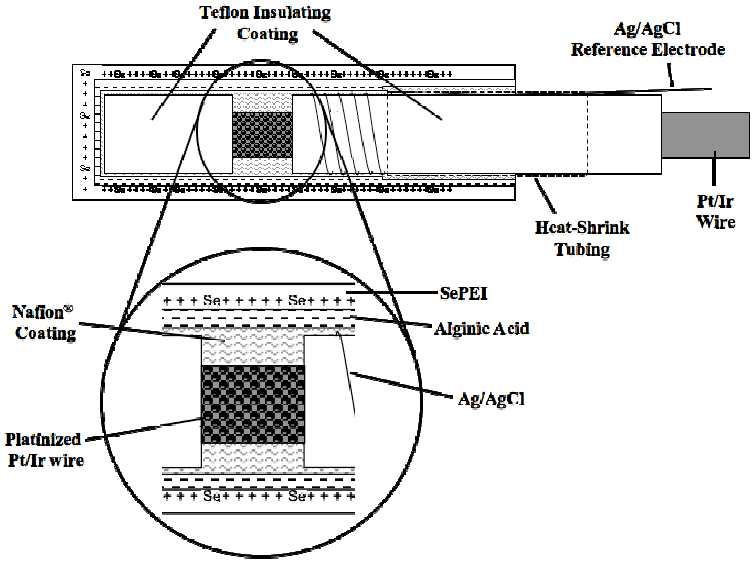

Figure 4. Schematic of needle type NO/RSNO sensor developed to measured NO/RSNO levels in SQ fluid.

3) Preparation of Needle-Type NO and RSNO Sensor: The development of needle-type NO and RSNO sensors was initiated to ultimately determine RSNO presence in the subcutaneous layer. The current design of the NO/RSNO sensor, based on a needle type electrochemical glucose sensor described by Bindra *et al.* [8], is illustrated in Figure 4 (o.d., = 0.3 mm). First, a Teflon-coated Pt/Ir wire is cut using a surgical blade to expose a section ~1 mm in length to serve as the sensing area. This exposed region is then platinized. A homemade Ag/AgCl reference electrode is then wrapped around the Teflon-insulated portion of the wire and bound to the wire using heat-shrink tubing. The sensing area and the reference electrode is then coated with Nafion® by dipping the sensor in a 1.25 wt% solution of Nafion® for 5 s and then drying at ~195 ^{o}C for 4 min. This dipping procedure is repeated 5 times. This is the needle-type NO sensor currently used (e.g., Figure 4 device but w/o the SePEI and alginic acid layers). The sensor exhibits good response to NO, with sensitivity of 30-60 nA/µM and a detection limit of 5 nM. To create the RSNO sensor, the LbL approach described above is now being tested to immobilize RSe on the surface of the Nafion layer (which already a polyanion) to catalyze the decomposition of RSNOs to NO. We are currently in the process of optimizing the number of bi-layers of the RSe-PEI with the alginate polyanion, etc.

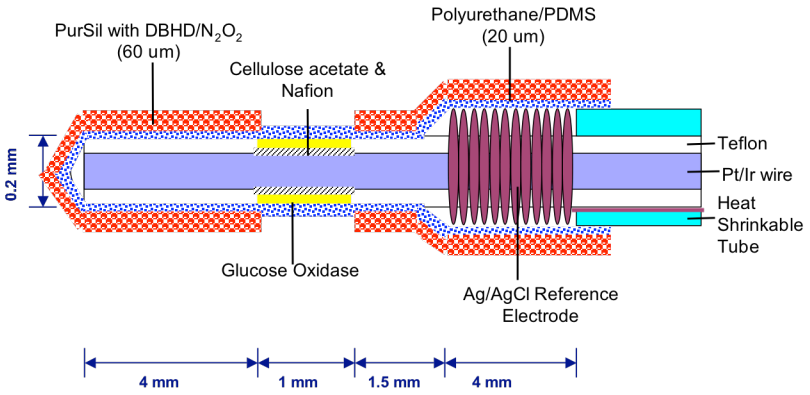

Figure 5. Schematic of new design for needle type electrochemical glucose sensor with longer-term NO release capability. Thicker coating of NO releasing polymer is over entire surface of sensor, except small window where enzymatic layer resides.

4) Fabrication of Functional Needle-type Glucose Sensors: In last year's report, we provided data indicating that we can prepare needle type amperometric glucose sensors (see Figures 4/5) with excellent linear response to glucose over the physiologically relevant concentration range. While our long-term goal remains to use the new NO generating coatings on these sensors and test in the SQ fluid of rats, given the repeatedly demonstrated clear benefits of NO releasing polymers in preventing inflammatory response in all the animal studies reported in Section 2, above, we decided to assess whether we can reconfigure needle type glucose sensors to achieve longer-term NO release capability. This has now been

accomplished using a relatively simple design that allows us to coat thicker layers of NO release polymers (polyurethanes/polydimethylsiloxane (PDMS) copolymers doped with lipophilic diazeniumdiolates [9]). In the past, efforts to do this have reduced the ability to detect glucose, since thicker outer coatings doped with the NO donors have limited permeability to glucose. The new design (see Figure 5) overcomes this problem. The thicker coating does not cover a 1 mm window of the sensor directly where the glucose oxidase enzyme is deposited over the exposed Nafion coated platinum electrode. However, the remaining surfaces of the needle sensor are coated completely with the PU/PDMS material doped with the NO donor. We have found that this new configuration gives excellent glucose response, while the entire sensor emits NO for up to 3 days at physiologically relevant fluxes. A recent paper by Hetrick et al. [10] suggests that even short-term NO release for such a 2-3 d period could decreased foreign body response over extended time periods (6 weeks). If so, this simple NO release glucose sensor design could provide the dose of NO needed to achieve the desired behavior *in vivo*.

We also still plan to study the effect of using different outer polymer coatings that possess the immobilized Cu(II) and RSe sites for generating NO from RSNO species for preparing needle type glucose sensors. Indeed, now that the PU material has been modified with immobilized Cu(II)-cyclen complex, this material will be first material to be tested for preparing NO generating glucose sensors. Again, using the new configuration shown in Fig. 5 will eliminate concerns that Cu(II)-cyclen-PU outer coating can effect glucose response, since only plain PU will coat the actual glucose sensing layer, and the NO generating material will be present over the remainder of the device.

KEY RESEARCH ACCOMPLISHMENTS (during year 2):

- Synthesized Cu(II)-cyclen compound covalently attached to a polyurethane matrix and demonstrated that this new material is able to catalyically generate NO from RSNO species at physiological pH values.

- Synthesized new organoselenium compounds attached to PEI, and used this new polymeric material to create a LbL coating method to place NO generating RSe sites on the surface of almost any sensor surface.

- Studied the use of organotellurium compounds as catalysts for NO generation, and demonstrated, for the first time, that covalently linked RTe species can indeed generate NO from RSNOs in a manner quite similar to RSe species.

- Obtained contradictory *in vivo* data on effectiveness of NO generating immobilized Cu(II) coatings for reducing inflammatory response for SQ implants in rats. Tests are presently being repeated.

- Constructed first NO and RSNO needle type sensors for ultimate use in measuring NO/RSNO levels in SQ fluid of rats.

- Devised new NO release SQ glucose sensor design, in which NO release can be achieve for prolonged periods, without compromising glucose response characteristics.

REPORTABLE OUTCOMES

Conference presentations:

-J. Yang, M. E. Meyerhoff, "Layer-by-Layer Assembly for Nitric Oxide Generation Based on Catalytic Decomposition of S-Nitrosothiols by Organoselenium Species," 2007 Annual Meeting of Society for

Biomaterials, April 18, 2007, Chicago, IL.

-Y. Wu, W. Cha, S. Hwang, F. Zhang, M. E. Meyerhoff, "Determining S-Nitrosothiol Concentrations in Whole Blood via Electrochemical Sensors Based on Immobilized Catalysts." Second International Conference on Role of Nitrite in Physiology, Pathophysiology and Therapeutics, NIH, September 6-7, 2007, Bethesda, MD.

Publications:

-M. Frost and M. E. Meyerhoff, "*In Vivo* Chemical Sensors: Tackling the Biocompatibility Issue," Anal. Chem., 78,7370-7377 (2006).

-S. Hwang and M. E. Meyerhoff, "Organoditelluride-Medidated Catalytic S-Nitrosothiol Decomposition," J. Mater. Chem, 17, 1462-1465 (2007)

-Y. Wu and M. E. Meyerhoff, "Nitric Oxide Releasing/Generating Polymers for the Development of Implantable Chemical Sensors with Enhanced Biocompatibility, Talanta, in press, 2007

-S. Hwang and H. E. Meyerhoff, "Organoditelluride-Tethered Polymers that Spontaneously Generate Nitric Oxide when in Contact with Fresh Blood," J. Mater. Chem., submitted, 2007.

CONCLUSIONS

During the past year, a new material was synthesized and characterized that utilizes Cu(II)-cyclen compound covalently attached to a commercial biomedical grade polyurethane. This material generates NO from RSNOs species present in solution at physiological pH as detected chemiluminescence. Initial *in vivo* evaluation of this material will begin shortly, as a coating on implanted SQ sham devices. In addition, new immobilized organoselenium and organotellurium catalysts have been examined for generation of NO from endogenous RSNO species. These new catalysts appear to be promising alternates for *in vivo* testing, should the Cu(II)-ligand based coating yield unacceptable results. Indeed, further *in vivo* studies using a lipophilic Cu(II)-cyclen compound blended into a biomedical grade polyurethane have yielded contradictory biocompatibility results compared to Year 1 data. There may have been a error in labeling the implants, and further animal experiments are underway to reach a final conclusion on the effectiveness of NO generating Cu(II)-ligand doped polymers in reducing inflammatory response when implanted sub-Q in rats. However, most data obtained thus far for the sham-sensor implants to test inflammatory response over 1week period has shown that NO release polymeric coatings consistently exhibit the greatest degree of biocompatibility (reduced migration of inflammatory cells to implant site). Hence, use of NO release coatings can serve as a backup strategy to the NO generating coatings, only if prolonged NO release can be achieved. During the past year, a new implantable glucose sensor design has been prepared in which the NO release coatings cover 95% of the sensor surface with an increased thickness to yield longer term NO release from the entire device but without altering the analytical response characteristics toward glucose. Continued testing of this new configuration will take place in the coming year, along with animal studies with the implantable RSNO/NO sensors to assess the levels of RSNO species in the sub-Q fluid of rats.

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